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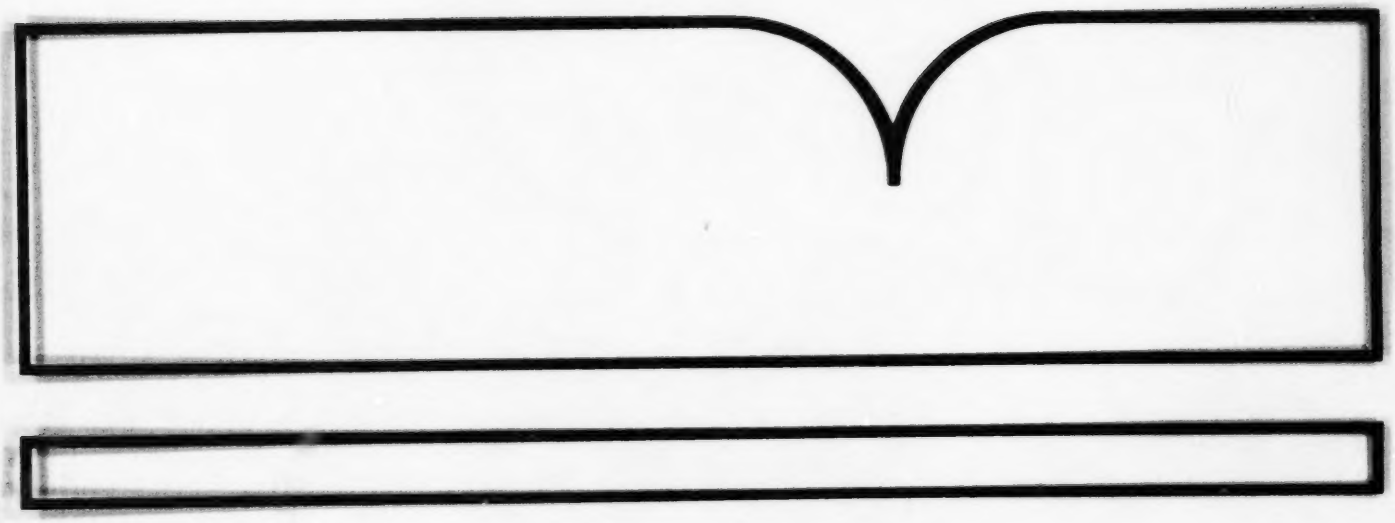

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16. ABSTRACT

We have evaluated a group of short-term bioassays to identify those that may be suitable for screening large numbers of diverse hazardous industrial wastes for genotoxicity. Fifteen wastes (and dichloromethane extracts of these wastes) from a variety of manufacturing processes were tested for mutagenicity in Salmonella typhimurium strains TA98 and TA100 with and without Aroclor 1254-induced rat-liver S9. Ten of these wastes were fed by gavage to F-344 male rats, and the raw urines were assayed for mutagenicity in the presence of β-glucuronidase in strain TA98 with S9. Six of these urines were extracted by C_{18}/methanol elution, incubated with β-glucuronidase, and evaluated in strain TA98 with S9 and β-glucuronidase. Fourteen of the wastes were examined for their ability to induce prophage lambda in Escherichia coli in a microsuspension assay. A second set of wastes, consisting of four industrial wastes, were evaluated in Salmonella and in a series of mammalian cell assays to measure mutagenicity, cytogenetic effects, and transformation. Our results suggest that the Salmonella assay using either strain TA98 or TA100 in the presence of S9 and the phage-induction assay in the presence of S9 were the most sensitive to the genotoxicity of these diverse waste types. Neither urinary mutagenicity nor any of the mammalian cell assays appear useful at this time for routine screening purposes.

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ASSESSMENT OF HAZARDOUS WASTES FOR GENOTOXICITY

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SUMMARY

We have evaluated a group of short-term bioassays to identify those that may be suitable for screening large numbers of diverse hazardous industrial wastes for genotoxicity. Fifteen wastes (and dichloromethane extracts of these wastes) from a variety of manufacturing processes were tested for mutagenicity in Salmonella typhimurium strains TA98 and TA100 with and without Aroclor 1254-induced rat-liver S9. Ten of these wastes were fed by gavage to F-344 male rats, and the raw urines were assayed for mutagenicity in the presence of β-glucuronidase in strain TA98 with S9. Six of these urines were extracted by C_{18}/methanol elution, incubated with β-glucuronidase, and evaluated in strain TA98 with S9 and β-glucuronidase. Fourteen of the wastes were examined for their ability to induce prophage lambda in Escherichia coli in a microsuspension assay. A second set of wastes, consisting of four industrial wastes, were evaluated in Salmonella and in a series of mammalian cell assays to measure mutagenicity, cytogenetic effects, and transformation. Our results suggest that the Salmonella assay using either strain TA98 or TA100 in the presence of S9 and the phage-induction assay in the presence of S9 were the most sensitive to the genotoxicity of these diverse waste types. Neither urinary mutagenicity nor any of the mammalian cell assays appear useful at this time for routine screening purposes.

INTRODUCTION

In the United States, wastes are classified as hazardous if they possess one of a number of physical characteristics or if they are composed of certain industrial process wastes or contain specifically regulated chemicals (ref. 1). Guidelines for characterizing the genotoxicity of hazardous wastes have not yet been promulgated; however, knowledge of the genotoxic potential of a hazardous waste can augment the available chemical characterization and provide relevant information regarding potential chronic health effects.

Because hazardous wastes may contain a wide array of chemical mutagens, and because no single bioassay will detect all chemical classes of mutagens, several short-term assays with different genetic endpoints may be advantageous for screening hazardous wastes for genotoxic potential. Consequently, combinations of short-term assays have been proposed as possible screening batteries for hazardous wastes (ref. 2). Nonetheless, most investigations of the genotoxicity of hazardous wastes have not used a battery of assays. Instead, most studies have used only the Salmonella assay (refs. 3,4), although mammalian cells and other eukaryotic assays have been used to a limited extent (refs. 5-7).

DISCLAIMER

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This report reviews the efforts of our laboratory to determine the usefulness of a variety of short-term bioassays for assessing the genotoxic potential of hazardous industrial wastes. Most of the wastes we evaluated had been partially chemically characterized, permitting comparisons of biological activity and chemical composition. The chemical analysis indicated that many of the wastes contained high concentrations of carcinogenic metals, chlorinated compounds, and solvents that are detected poorly by the Salmonella assay (refs. 8-10). Thus, in addition to the Salmonella assay, we included a prophage-induction assay in Escherichia coli that may be more sensitive than Salmonella for these classes of compounds (refs. 11,12). Because in vivo mammalian metabolism may be a critical factor in the generation of mutagenic metabolites from complex hazardous wastes, we also studied the urinary mutagenicity of rats fed hazardous wastes by gavage. Finally, because most batteries include mammalian cell assays, we evaluated a series of such assays and compared the results to those obtained with Salmonella for a set of four diverse hazardous wastes. The advantages and disadvantages of testing crude versus extracted waste samples are discussed along with some of the problems encountered when trying to select appropriate test methodologies for a wide variety of hazardous waste types.

MATERIALS AND METHODS

Wastes and waste extracts

Two sets of hazardous wastes were used for these studies (Table 1). Set 1 was obtained from Edward L. Katz, Hazardous Waste Engineering Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. Three of the waste samples (A, B, and C) were from three different manufacturing processes (petrochemical, pharmaceutical, and plastics). The remaining 12 samples (D through O) were from four commercial hazardous waste incineration facilities that burn a mixture of hazardous wastes from a variety of industrial sources. Each waste was analyzed for the presence of a limited number of priority organics and/or metals identified in the U.S. EPA Appendix VIII list of priority pollutants (ref. 13). DCM extracts were prepared as described (ref. 14), solvent exchanged into DMSO, and both the crude wastes and waste extracts were tested in the Salmonella assay. Only the crude wastes were evaluated in the phage-induction assay and fed by gavage to rats to determine the mutagenicity of the urine.

The second set of hazardous wastes (Table 1) consisted of four wastes from four different industries: coke plant, herbicide manufacturing, pulp and paper mill, and oil refining. These wastes were obtained from Dr. M. McKown, Battelle Columbus Laboratories, Columbus, Ohio. DCM extracts were prepared as described (ref. 15) and solvent exchanged into DMSO for bioassay. These four waste extracts were tested in Salmonella and in the mammalian cell assays.

TABLE 1

Physical descriptions of hazardous wastes

Wastes	Description
Waste Set 1	
A	Black, thin oil from a petrochemical manufacturing plant
B	Black, oily liquid from a pharmaceutical manufacturing plant
C	Black, pourable tar produced from a plastic manufacturing plant
D and E	Composite of aqueous wastes; watery liquid with red oil drops
F and G	Composite of organic wastes; biphasic gray sludge with reddish-brown liquid
H and I	Composite of aqueous wastes; thin, gray slurry
J	Composite of organic wastes; gray, thick liquid with suspended solids
K	Similar to K, but lighter in color and thinner
L and M	Composite of organic wastes; black, thin, pourable tar
N and O	Composite of aqueous wastes; clear, watery liquid
Waste Set 2	
P	Light-brown liquid with suspended solids from coke plant
Q	Reddish-brown clear liquid from herbicide manufacturing plant
R	Gray-brown semi-solid with wood chips from pulp and paper mill
S	Dark liquid with brown-black flocculant and oil drops from oil refining plant

Urine and urine extracts

F-344 male rats were dosed with 4 different doses of waste C for 10 days before collecting 24-h urine from 3 rats per dose. However, the available amounts of waste samples permitted the use of this protocol for only waste C. For nine other wastes, a single dose of the crude waste was administered by gavage to 70-day-old male F-344 rats. All urines were collected on dry ice for 24 h, centrifuged, filter sterilized, and frozen at -20°C. All 10 raw urines were tested for mutagenicity in Salmonella TA98 with S9 and β-glucuronidase. In addition, six of these raw urines were also extracted and tested as follows.

1 ml of β-glucuronidase (Sigma Type VII from E. coli) at a concentration of 1000 units/ml of potassium phosphate buffer (0.15 M, pH 7.4) was added to 2-10 ml of thawed urine, and the mixtures were incubated with shaking for 1 h at 37°C. Then, each mixture was poured through two serially connected Sep-Pak® C_{18} cartridges (Waters Associates, Milford, MA) followed by water. Concentrates were then eluted with methanol, the methanol was evaporated, and the residue was solvent exchanged into a volume of DMSO to produce 5 X concentrates, which were stored at -20°C until bioassayed.

Salmonella assay

The Salmonella plate-incorporation assay was performed as described (ref. 16). Wastes and waste extracts were assayed at least twice, each in either

duplicate or triplicate, in strains TA98 and TA100 in the presence and absence of Aroclor 1254-induced Sprague-Dawley rat liver S9 (~1 mg of protein/ plate) prepared as described (ref. 16). Urines and urine extracts were assayed in strain TA98 in the presence of S9 and β-glucuronidase (1000 units/plate). Raw urine and urine extracts were tested twice, each in singlet. A dose-related increase in the number of revertants/plate was considered a positive response.

Phage-induction assay

The Microscreen phage-induction assay developed by Rossman et al. (ref. 11) was performed using modifications described previously (ref. 17). The two bacterial strains used for this assay are derived from E. coli B/r. $WP2_s(\lambda)$ is a lambda lysogen of $WP2_s$ (trpE, uvrA); SR714 (trpE; $uvrD_3$) is the indicator strain. The lysogenic strain was exposed overnight to various dilutions of the crude wastes both in the presence and absence of S9. Following exposure, each suspension was sampled for the presence of lambda particles by plating onto the indicator strain. The criterion for a positive response was an increase in the number of induced plaque-forming units per plate that reached or exceeded the upper limit of the 99% confidence interval based on the negative controls.

Mammalian cell assays

Because of the limited amount of DCM extract of each of the four wastes used in the mammalian cell assays, and because of the cost of performing a set of such assays with each extract, it was not possible to perform the assays according to currently accepted protocols or established guidelines. Instead, limited protocols were used that required a minimum number of steps and amount of sample to permit an extract to be identified as a presumptive positive or negative. In order to conserve sample, the dose range for each extract was estimated for all of the assays by performing a preliminary cytotoxicity study in Chinese hamster ovary (CHO) cells. The assays were then performed only once with only a few doses of extract and in the presence of S9. Otherwise, the assays were performed essentially as described below.

The L5178Y/$TK^{+/-}$ -3.7.2C mouse lymphoma assay was performed in the presence of S9 as described (ref. 18). Cytogenetic effects induced by the four waste extracts in the presence of S9 were determined by scoring for chromosomal aberrations and sister chromatid exchanges (SCEs) in CHO-WBL cells as described (ref. 19). The ability of the four waste extracts to induce morphological transformation in BALB/c-3T3 cells was determined as described (ref. 20). Metabolic activation was provided by X-irradiated rat liver cells, and 12-O-tetradecanoyl-13-phorbol acetate (TPA) was used to promote the formation of the transformed phenotype.

RESULTS AND DISCUSSION

The quantitative results of our genotoxicity studies of these wastes and waste extracts have been published elsewhere (refs. 14,15,17); the qualitative responses are summarized here. Table 2 summarizes the mutagenic responses in Salmonella of the first set of wastes and waste extracts, the mutagenic responses in Salmonella of the the urine or urine extracts from rats fed these wastes, and the responses of these wastes in the phage-induction assay. Table 3 summarizes the genotoxic responses of the second set of wastes in Salmonella and in the mammalian cell assays.

TABLE 2

Genotoxic responses of crude wastes/waste extracts and raw urines/extracts

	Mutagenic responses in Salmonella										Phage-induction responses	
	Crude wastes				Waste extracts							
	TA98		TA100		TA98		TA100		Urines[a]			
Wastes	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	Raw	Extracts	+S9	-S9
C	-	-	+	+	+	-	+	-	+	+	+	+
L	+	-	-	-	-	-	-	-	+	+	+	+
M	+	-	-	-	-	-	-	-	+	+	+	+
G	+	+	-	-	-	-	-	-	-	-	+	+
O	+	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	NT[b]	NT
H	-	-	-	-	+	+	-	-	-	NT	+	+
J	-	-	-	-	-	+	-	-	-	NT	+	-
K	-	-	-	-	-	-	-	-	-	NT	-	-
B	-	-	-	-	NT	NT	NT	NT	-	NT	+	+
A	-	-	+	+	-	-	+	+	NT	NT	+	+
F	-	-	+	+	-	-	-	-	NT	NT	+	+
D	-	-	-	-	-	-	-	-	NT	NT	+	+
I	-	-	-	-	-	-	-	-	NT	NT	+	+
N	-	-	-	-	-	-	-	-	NT	NT	-	-

[a]Urines tested in strain TA98 in the presence of S9 and β-glucuronidase.
[b]NT, not tested.

TABLE 3

Genotoxic responses of four waste extracts in Salmonella and mammalian cells

	Mutagenicity in Salmonella				Genotoxicity in mammalian cells			
					Mutagenicity in	Cytogenetic effects		Transformation in
	TA98		TA100				Chromosomal	
Waste extracts	+S9	-S9	+S9	-S9	L5178Y/TK$^{+/-}$	SCEs	aberrations	BALB/c-3T3
P	+	-	+	-	+	+	+	-
Q	+	-	-	-	+	-	-	-
R	+	-	-	-	+	+	-	-
S	-	-	-	-	+	+	-	-

Liquid extraction, as opposed to solid-phase extraction, was the only type of extraction procedure that was suitable for these diverse wastes. However, as discussed previously (ref. 14), DCM may not be a suitable solvent for all types of wastes. Table 2 shows that DCM failed to extract mutagenic activity from five wastes (L, M, G, F, O) that were mutagenic in their crude, unextracted form. Nearly 80% of these wastes would have been detected as mutagenic if only the crude wastes had been tested. The additional time and expense required to prepare organic extracts of these wastes did not produce extracts that yielded much additional information that was not obtainable from the crude wastes. However, not all wastes can be tested directly due to microbial contamination or physical state, e.g., viscosity, pH, etc. Thus, extraction/fractionation procedures will be necessary for some, if not most, hazardous wastes in order to examine their biological activity. An innovative approach involving fractionation by thin-layer chromatography coupled with the Salmonella assay has been shown to be useful with some wastes (ref. 3).

Judicious selection of a test matrix (and battery) is required in order to screen hazardous wastes in a cost-effective manner without an unacceptable loss of detection capability. For example, all of the wastes and waste extracts that were mutagenic in Salmonella in Table 2 would have been detected if they had been tested only in the presence of S9 (Table 2), reducing the testing matrix in half. Considering both the crude wastes and waste extracts, only two wastes (A and F) were mutagenic solely in strain TA100. Thus, if the crude wastes and extracts had been studied only in TA98 in the presence of S9, nearly 80% of the mutagenic wastes identified with the present matrix would have been identified.

Based on the wastes tested here, the urinary mutagenesis assay did not provide any additional information beyond that obtained from testing the wastes or waste extracts directly in the Salmonella assay. Only three of the 10 wastes tested produced mutagenic raw urine, and all three of these wastes were mutagenic when tested directly in Salmonella (Table 2). Extracting the urines by means of C_{18}/methanol elution did not identify a urine as mutagenic that was not identified as mutagenic from studies with raw urine (Table 2). Considering the time and expense of performing the urinary mutagenesis assay, this assay was not a useful adjunct to testing the wastes or waste extracts directly for mutagenicity.

The phage-induction assay in E. coli detected five crude waste samples that were not mutagenic in Salmonella (Table 2). As described in the Introduction, the Microscreen phage-induction assay has been shown to detect some carcinogenic metals and chlorinated organics and solvents that are not mutagenic in Salmonella. Metals and compounds of these types are present in most of the waste samples studied here (ref. 13), and the ability of some of these

compounds to induce prophage may account for the detection by the phage-induction assay of the five additional waste samples. Accumulating evidence indicates that prophage induction (and the SOS response in general) is a broader genetic endpoint than reverse mutation in bacteria (refs. 21-23), making it especially useful for screening chemically diverse wastes.

A comparison of the genotoxic responses of DCM extracts of four wastes in the Salmonella assay to their responses in a set of mammalian cell assays indicates that the inclusion of mammalian cell assays may not have improved significantly the ability to detect the genotoxicity of the wastes beyond that afforded by the Salmonella assay alone (Table 3). The oil refining waste was the only waste detected by the mammalian cell assays that was not detected by the Salmonella assay. The BALB/c-3T3 transformation assay did not detect any of the extracts as positive, even though all four were genotoxic in one or more assays. Based on these limited results, it appears that this assay may not be useful for screening hazardous wastes.

Currently, there is only a small data base on the use of mammalian cell assays with complex mixtures, let alone with hazardous wastes. Perhaps some of the reasons for this are that mammalian cell assays are difficult to use with toxic complex mixtures and are more costly and time consuming to perform than microbial assays. Recently, several studies (refs. 24,25) have shown that mammalian cell assays may not provide much additional detection capability than that afforded by the Salmonella assay for pure compounds. Our results with these hazardous wastes suggest that the same may be true for complex mixtures.

Our main purpose in these studies has been to determine which individual bioassays or groups of bioassays could serve as inexpensive, rapid screening tools to assess the genotoxicity of a large number of chemically different industrial wastes. Although diverse, the hazardous wastes we have studied here cannot be considered to represent the "universe" of wastes. In addition, there are many other bioassays that we have not yet examined that may be useful for screening hazardous wastes. Given these limitations, however, our studies suggest that the Salmonella assay using either strain TA98 or TA100 in the presence of S9 or the phage-induction assay in the presence of S9 may be useful in screening wastes for genotoxicity.

Both government (refs. 26-28) and industry (refs. 2, 29) have recognized the important role that short-term tests could play in the toxicological assessment of hazardous wastes. We have reported here on the use of only genotoxicity bioassays to evaluate hazardous wastes; however, wastes may induce other biological effects, such as hepatotoxicity or neurotoxicity. Additional investigations are needed to explore the effects of hazardous wastes at these and other toxicological endpoints. The results presented here suggest that short-

term genotoxicity bioassays may be useful for detecting the potential genotoxicity of wastes and may aid in the identification of some wastes as hazardous.

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